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Simple and rapid detection of human enterovirus 71 by reverse-transcription and loop-mediated isothermal amplification: cryopreservation affected the detection ability☆

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Abstract

Human enterovirus 71 (EV71) is the primary pathogen of hand, foot, and mouth disease (HFMD). EV71 infection may lead to neurologic damage, with higher incidence of fatality compared with other HFMD pathogens. An effective drug or vaccine against EV71 infection is currently unavailable. It is desirable to determine the pathogen of HFMD accurately and quickly for early treatment. In the current study, reverse-transcription and loop-mediated isothermal amplification (RT-LAMP) technology were developed to detect EV71. The efficacy of detecting EV71 was compared with regular nested reverse-transcription polymerase chain reaction (RT-PCR). After detecting 108 clinical specimens, results showed that RT-LAMP can specifically detect EV71, but not Coxsackie virus A16, and exhibited a specificity of 100% and a sensitivity of 97.1%, which was higher than regular RT-PCR. The findings indicate that RT-LAMP is a practical method for EV71 diagnostic applications, particularly in small county institutes of medical service. The detection ability of RT-LAMP was significantly affected by cryopreservation as the clinical specimens were repeatedly subject to freezing and thawing treatments.

Keywords: EV71; Reverse-transcription and loop-mediated isothermal amplification; Cryopreservation

1. Introduction

Enterovirus 71 (EV71), first isolated in 1969 in California, is a major public health issue across the Asia Pacific region, Europe, and other continents. The virus mostly affects young children and causes hand, foot, and mouth disease (HFMD), which is generally a mild illness characterized by blisters and ulcers (Cardosa et al., 2003; McMinn, 2002; Solomon et al., 2010; Wu et al., 2010). However, unlike other HFMD pathogens, such as the Coxsackie virus and echovirus, EV71 infection could result in severe neurologic complications, including aseptic meningitis, encephalitis, acute flaccid paralysis, myocarditis, pulmonary edema, and hemorrhage, with high incidence of fatality (Chang et al., 2007; Huang et al., 1999). EV71 was recently reported as the primary pathogen of HFMD among young children in China (Yang et al., 2009; Zhang et al., 2009), and over 1.77 million HFMD cases were reported in 2010, 905 of which were fatal. This number is a 53.6% increase from that reported in 2009, during which over 1.15 million cases were reported and 353 cases were fatal (Ministry of Health, PR China).

EV71 is a small, single-stranded, positive-sense RNA virus, belonging to the family of Picornaviridae. The EV71

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genome, which is approximately 7.4 kb long, encodes a long polyprotein with a single open reading frame followed by a poly A tail. The single polyprotein is flanked by untranslated regions at both the 5′ and 3′ ends, and can be divided into 3 different genomic regions. The P1 genomic region encodes for structural proteins (VP1 to VP4), while the P2 and P3 genomic regions encode for nonstructural proteins 2A, 2B, 2C, 3A, 3B, 3C, and 3D (Cardosa et al., 2003; Solomon et al., 2010; Wu et al., 2010). Research on EV71 drugs and vaccines is ongoing and some progress has been achieved (reviewed by Lee and Chang, 2010; Wu et al., 2010), and the potential receptors for EV71 infection were also identified (Nishimura et al., 2009; Yamayoshi et al., 2009). However, an effective drug or vaccine against EV71 infection is currently unavailable. Thus, determination of the pathogen of HFMD for an early treatment is desirable.

Recently, a novel method of DNA amplification, known as loop-mediated isothermal amplification (LAMP) of a target nucleic acid, was reported by Notomi et al. (2000). LAMP is a 1-step amplification reaction that amplifies a target DNA sequence with high sensitivity and specificity under isothermal conditions by a DNA polymerase (Bst polymerase) and a set of primers. The addition of reverse transcriptase makes it possible to amplify DNA from RNA sequences (RT-LAMP). This method has already been applied to the detection of several RNA viruses, such as Norovirus (Iturriza-Gomara et al., 2008), avian influenza virus H5N1 (Imai et al., 2006, 2007), and severe acute respiratory syndrome coronavirus (Poon et al., 2005). Here we sought to determine whether or not RT-LAMP could be applied to EV71 amplification and determine its diagnostic performance in clinical specimens.

2. Material and methods

2.1. Viral RNA preparation and amplified by regular RT-PCR

Clinical stool specimens were collected from patients identified with HFMD and registered in Children's Hospital of Fudan University, Shanghai. A total of 0.2 g of stool from each patient was thoroughly suspended in 1.0 mL PBS buffer, after which viral RNA was extracted with Trizol (Invitrogen, USA) following the manufacturer's instructions. Five microliters of extracted RNA (a total of 20 μL) was reverse transcribed (RT) with M-MLV reverse transcriptase (Takara, Japan) in a volume of 20 μL consisting of 50 ng hexamer, RNAguard (Pharmacia, USA) at 400 U/mL, 200 U of M-MLV, 1 μL of 0.1 M DTT, and 2 μL of 10 mmol/L dNTP mixture. The RT reaction was incubated at 37 °C for 1 h and inactivated at 75 °C for 10 min. The RT products were then amplified by polymerase chain reaction (PCR). The PCR mixture, in a volume of 25 μL, consisted of 5 μL of RT products, 2.5 μL of 10 × PCR buffer, 2.5 μL of 25 mmol/L $MgCl_2$, 2.5 μL of 2.5 mmol/L dNTP mixture, 1 μL of sense primer (10 μM), 1 μL of antisense primer (10 μM), and 0.25 μL of DNA Taq polymerase (5 U/μL). PCR was performed via a touch-down program: 94 °C for 4 min, after 10 cycles of 94 °C for 45 s, 60 °C for 45 s (decreasing at a rate of 0.5 °C/cycle for annealing in the next 9 cycles), 72 °C for 45 s, and then continued for another 20 cycles at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s, and, finally, at 72 °C extended for 7 min on a PCR machine (Eppendorf Mastercyler personnel 5333). Nested PCR was continued with 1 μL of the previous PCR products by inner sense and antisense primers. The amplified products were detected by running with 2% agarose gel and staining with 0.5% ethidium bromide (EB).

2.2. Viral RNA amplified by RT-LAMP

Five microliters of extracted RNA (the same amount for regular RT-PCR) was taken for RT-LAMP amplification in a 25-μL mixture consisting of Bst DNA polymerase (New England Biolab) 8 U, 10× thermal buffer 1.0 μL, AMV reverse transcriptase (Fermentas) 5 U, 5× AMV first-strand buffer 4.0 μL, 25 μM betaine (Sigma, USA) 1.0 μL, 2.5 mmol/L dNTP mixtures 5 μL, 0.1% Triton X-100 0.25 μL, and a primer mixture, including 0.2 μM F3 and B3, and 1.6 μM FIP and BIP. RT-LAMP primer sets were designed using the Primer Explorer V3.0 online software (http://primerexplorer.jp/v3_manual/index.html) and manually selected according to the principle for RT-LAMP (Table 1 and Fig. 1). After 60 °C for 1 h, the polymerases in the reaction mixture were inactivated at 80 °C for 5 min, and then 5 μL of RT-LAMP products was detected by running with 2% agarose gel and staining with EB.

2.3. RT-LAMP products identified by Southern blot hybridization and sequencing

To confirm the facticity after amplification by RT-LAMP, 10 μL of RT-LAMP products was run with 1% agarose gel

Table 1
Primers for RT-LAMP and regular nested RT-PCR amplification of EV71

Detection methods	Primer name	Location (FJ713317)	Direction	Sequences
RT-LAMP	EV71-F3	2577–2597	External sense	5-act cca agc tgc tga aat tgg-3
	EV71-B3	2778–2797	External antisense	5-gca ttt gtg cgt aac ctg tt-3
	EV71-FIP	2650–2669/2610–2629	Inner sense	5-*tca gct gtg ctg tgc gag tt*t gct agt gac gag agc atg a-3
	EV71-BIP	2687–2710/2743–2763	Inner antisense	5-*tct tca gca gag cgg gat tag ttg g*tt ggc ata acc att tgg gtt-3
Nested RT-PCR	EV71-F	2758–2781	Sense	5-gcc aac tgg gac ata gat ata ac-3
	EV71-R1	3312–3333	External antisense	5-ccc aag agt agt gat cgc tgt-3
	EV71-R2	3137–3155	Inner antisense	5-ccc aca gtc cgc act gag-3